EXHIBIT 3

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PROGNOSTIC SIGNIFICANCE OF BAG-1 EXPRESSION IN NONSMALL CELL LUNG CANCER

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The purpose of this study was to evaluate the expression of BAG-1 in a cohort of patients with nonsmall cell lung cancer (NSCLC). The intensity and subcellular distribution of BAG-1 expression were correlated with overall survival. Tumor samples were collected from 85 patients diagnosed with NSCLC between 1993–1995 in St. John's, Newfoundland. Expression of BAG-1 was determined by immunohistochemistry using polyclonal anti-BAG-1 antibody. There was significant variation in the immunohistochemical staining patterns of BAG-1, including nonstaining and staining of either the cytoplasm, nucleus or both. Univariate Cox regression analysis showed that those patients whose tumor overexpressed BAG-1 had a significant reduction in the risk of death (hazard ratio = 0.53, $p = 0.03$). The survival advantage of patients with BAG-1 overexpression tumor was also demonstrated by Kaplan-Meier analysis and log-rank tests (median survival 30.10 months versus 17.04 months, $p = 0.05$). In addition, multivariate Cox regression analysis showed that patients whose tumor exhibited intense cytoplasmic staining had a further reduction of the risk of death (hazard ratio = 0.42, $p = 0.03$) and this effect was independent of age, stage and histology. All stages were included in the analysis. Our preliminary data strongly indicate that further investigation is warranted to better define the role of BAG-1 as an independent prognostic factor in NSCLC.

Key words: *BAG-1/RAP46; apoptosis; lung cancer; prognosis*

Lung cancer is among the most common malignancies in the Western world and is the leading cause of cancer deaths in both men and women.[1] Diagnosis is often delayed, with 70% of patients presenting with advanced disease, and an overall 5-year survival rate of less than 15%.[2] Clinicopathologic features such as tumor size, nodal status and age have been shown to have prognostic importance in patients with resectable nonsmall cell lung cancer (NSCLC). Survival for stage I–II disease remains low even after complete surgical resection, with 40–50% of patients relapsing and experiencing 5-year survival of only 50–60%.[3] Adjuvant therapy in this patient group is under investigation, and many early trials are negative.[4] Efforts are ongoing to identify other prognostic factors which might enable the targeting of adjuvant therapies to appropriate high-risk subgroups.

Pulmonary carcinogenesis appears to evolve through a multistep process involving induction of oncogenes (such as *Ras*, *Myc*, *c-erbB-2* and *Bcl-2*) and loss of tumor suppressor genes (such as *RB* and *p53*).[5] Many investigators have attempted to correlate p53 expression with prognosis in NSCLC. Although mutations in the *p53* tumor suppressor gene have been demonstrated in up to 50% of patients with primary resected NSCLC,[6] data regarding the prognostic value of *p53* expression in NSCLC are conflicting. Several retrospective studies have suggested that expression of the *p53* gene carries poor prognosis. *p53* expression was an independent negative prognostic factor in a study limited to adenocarcinoma of the lung,[7] and in node-negative NSCLC, *p53* overexpression conveyed a poor prognosis.[8] Poor prognosis has been associated with other pro-apoptotic oncogenes. Expression of *Ras* oncogene product was shown to be an independent negative prognostic factor in NSCLC.[9–11] Conversely, others demonstrated that increased p53 expression increased survival significantly in a subgroup of lymph node-positive, nonsquamous lung cancers[12] and was related to longer metastasis-free survival.[13]

The prognostic value of anti-apoptotic gene expression in NSCLC has also been extensively investigated. Again, the results are inconclusive. Early investigations of Bcl-2 activity in NSCLC indicated a possible survival advantage.[14,15] Subsequent studies supported this finding, as Bcl-2 expression was correlated with increased disease-free survival in NSCLC and better overall survival in a subgroup of patients with squamous cell tumors. Bcl-2 expression has also been associated with increased overall survival in a group of NSCLC patients treated surgically for cure.[17] These findings were not confirmed in a recent series of similar retrospective studies.[18–20] Interestingly, even though a correlation between Bcl-2 activity and survival in NSCLC was not established, a study provided compelling evidence that metastatic disease was related to decreased Bcl-2 activity.[21] The relationship between anti-apoptotic Bcl-2 activity and prognosis in NSCLC remains unclear.

BAG-1 (also known as RAP46) is a recently identified Bcl-2-binding anti-apoptotic protein that may play a role in carcinogenesis and drug resistance.[22] BAG-1 is overexpressed in a variety of solid tumors such as breast, prostate and cervical cancers.[23,24] BAG-1 affects cell cycling and proliferation through multiple pathways. *In vitro*, BAG-1 was shown to bind Bcl-2 and increase its antiapoptotic activity.[25] Interaction of BAG-1 protein with retinoic acid receptors also inhibits apoptosis in cancer cells,[26] and interaction of BAG-1 with vitamin D receptors inhibits the vitamin D pathway.[27] BAG-1 protein also binds to hormone receptors and therefore may be of particular importance in hormone-sensitive cancers such as those derived from breast and prostate.[28] It also binds to hepatocyte growth factor (HGRF) and enhances protection from apoptosis by HGRF.[29] BAG-1 may play a key role in protein folding and trafficking via modulation of heat shock protein activity.[30] Finally, induction of BAG-1 as well as Bcl-2 probably represents key events in the anti-apoptotic function of cytokines, particularly interleukin-2 (IL-2).[31]

Deregulation of apoptosis has been implicated in the pathogenesis of lung cancer. BAG-1 interacts with Bcl-2, and its expression is upregulated by tumor-derived p53 mutants.[32] Because of its affect on apoptosis, BAG-1 may play an important role in lung cancer prognosis. Our preliminary finding of increased BAG-1 in lung cancer cell lines and tissues supports this hypothesis.[33] The

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present study attempts to test this hypothesis by retrospectively examining the relationship between immunohistochemically determined BAG-1 expression and overall survival in a cohort of patients with NSCLC.

PATIENTS AND METHODS

Patient inclusion and exclusion

Eighty-five patients diagnosed with NSCLC at St. Clare's Mercy Hospital in St. John's, Newfoundland between 1993–1995 were included in the study. These 85 patients were followed from the time of diagnosis to either March 1999 or until the time of death. The diagnosis of cancer was based on the original interpretation of the hematoxylin and eosin (H&E)-stained tissue produced at time of diagnosis. Patients with inadequate tissue for immunohistochemistry and incomplete staging were excluded from the study.

BAG-1 expression by immunohistochemistry

Paraffin-embedded lung tissue was sectioned and mounted on silane-coated slides. Deparaffinization was carried out in xylene. The slides were then rehydrated in a decreasing ethanol series ending in distilled water. Endogenous peroxidase activity was quenched using 0.6% hydrogen peroxide in 100% methanol for 15 min. Slides were placed in a humid chamber and incubated at room temperature for 60 min with 10% normal goat serum (D3002S, Dimension Laboratories, Ontario, Canada) in PBS containing 0.1% Triton X-100 (X-100, Sigma, St. Louis, MO) to block nonspecific staining. Sections were incubated at room temperature for 1 hr with BAG-1 polyclonal antibody (rabbit anti-mouse BAG-1 [C-16], sc-939, Santa Cruz Biotechnology, Santa Cruz, CA) at 250× dilution. The specificity of this polyclonal antibody to human BAG-1 was previously demonstrated in our laboratory.[34] This polyclonal antibody cross-reacts with BAG-1 isoforms such as BAG-1 p50, p46, p33 and p29[33] or BAG-2, BAG-3, BAG-4 and BAG-5.[35]

The sections were then incubated for 1 hr with a 200× dilution of biotinylated goat anti-rabbit IgG (BA-1000, Vector, Burlingame, CA). Slides were rinsed thoroughly with PBS between each of the above steps. Sections were then exposed to a diaminobenzadine (D-5673, Sigma) peroxidase substrate solution (DAB) for 5 min. The reaction was stopped using distilled water. Sections were then dehydrated in an increasing ethanol series and xylene and then coverslipped using Permount (SP153-100, Fisher Scientific, Fair Lawn, NJ). All slides were examined by 2 observers (including an independent pathologist) and categorized according to the subcellular location of staining (nucleus versus cytoplasmic) and the intensity (zero or no staining, light and intense staining). The observers reviewing the stained slides were blinded to the clinical information of the corresponding patients. Three slides from each tumor sample were stained. The percentage of positively stained cells within any tumor sample varied from section to section, and the slide with the highest percentage of positively stained cells (at least more than 25%) was chosen to score for BAG-1 expression.

Statistical analysis

Statistical analysis was performed using SPSS version 7.5.1 for Windows 95 (SPSS, Chicago, IL). Survival according to age, stage, histology and BAG-1 immunohistochemical staining was determined using Cox regression analysis and Kaplan-Meier plots. The level of significance for all analyses was set at 0.05.

RESULTS

Patient population

Eighty-five cases of NSCLC with complete staining and follow-up data were included in the study. Median duration of follow-up was 25.5 months. The percentage of males was 71.8. The median age was 63 years (range 38–80 years). Staging was done according to the 1997 ISS review for staging: 74.1% of patients were diagnosed with stage I and II disease and 25.8% of patients had stage III/IV disease. The majority of patients (81.2%) received surgical treatment, 4.7% received chemotherapy and 62.4% received radiation therapy. Adenocarcinoma was diagnosed in 68.2% of patients, 30.6% had squamous cell carcinoma and 1.2% had mixed cell carcinoma. Patients with advanced nonsmall cell lung cancer did not have paraffin-embedded lung cancer tissues and were therefore excluded from the study. More than 50% of the stage I patients were alive at the time of analysis. The characteristics of the patient populations are summarized in Table I, and the cumulative survival of this patient group stage by stage is illustrated in Figure 1.

BAG-1 expression by immunohistochemistry

Overall, BAG-1 expression was detected in 73% of NSCLC patients. There was significant variation in the immunohistochemical staining patterns of BAG-1, including nonstaining and staining of either the cytoplasm, nucleus or both. In many tissue specimens, the presence of malignant cells surrounded by normal lung parenchyma (as determined by H&E staining of each specimen) provided an excellent control for possible nonspecific staining. Furthermore, there was consistent staining of certain elements of normal lung tissue such as alveolar macrophages and pseudostratified or simple columnar epithelium. This is consistent with a recent study examining BAG-1 expression in normal human tissues.[36] The distinction between cytoplasmic and nuclear BAG-1 staining is demonstrated in Figure 2. Staining was also categorized according to intensity. Slides were described as either nonstaining, lightly staining or intensely staining, and each slide was reviewed independently by 2 examiners.

Statistical analysis

In analysis by logistic regression, BAG-1 expression was independent from other traditional prognostic factors such as age ($p = 0.71$), sex ($p = 0.84$), stage ($p = 0.35$) and histology ($p = 0.43$) (Table II). In the univariate Cox regression analysis, patients whose tumor overexpressed BAG-1 had a significant reduction in the risk of death with a hazard ratio of 0.53 and a p value of 0.03. Other factors that were correlated with patient survival included age ($p = 0.02$) and stage ($p < 0.01$) (Table III). When BAG-1 expression and survival were analyzed together with age, sex, stage and histology in a multivariate Cox regression analysis, patients whose tumor overexpressed BAG-1 had a trend toward reduced risk of death, with a hazard ratio of 0.58 ($p = 0.07$). The other independent prognostic factors that remained significant were stage ($p = 0.00$) and age ($p = 0.48$) (Table IV). BAG-1 became an independent prognostic factor (HR = 0.42, $p = 0.03$) when cytoplasmic BAG-1 expression was analyzed against age, sex, stage and histology in multivariate Cox regression (Table V).

TABLE I – CHARACTERISTICS OF THE PATIENT POPULATION

Characteristic	No.	%
No. of patients	85	100
Sex		
Male	61	71.8
Female	24	28.2
Age at diagnosis (yr)		
Range	38–80	
Mean	62.36	
Median	63	
Stage		
I	39	50
II	19	24.1
III	14	15.2
IV	13	10.6
Treatment		
Surgery	69	81.2
Chemotherapy	4	4.7
Radiation	53	62.4
Histology		
Adenocarcinoma	58	68.2
Squamous	26	30.6
Mixed	1	1.2

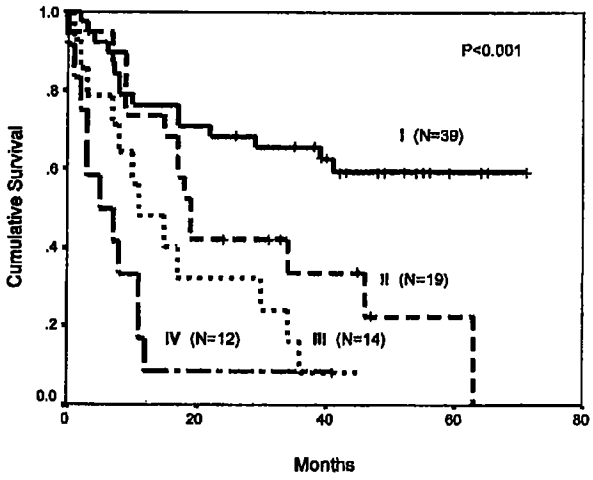

FIGURE 1 – Kaplan-Meier analysis for overall survival correlated with stage of tumors for 85 patients with NSCLC.

The survival advantage among patients with nonsmall cell lung cancer whose tumor overexpressed BAG-1 was also demonstrated in Kaplan-Meier analysis and log-rank tests (Fig. 3). Patients whose tumor overexpressed BAG-1 had a median survival of 30.10 months, compared with patients whose tumor did not overexpress BAG-1, with a median survival of 17.0 months ($p = 0.05$). When we further analyzed the impact of cytoplasmic versus nuclear BAG-1 expression on survival, patients whose tumor overexpressed cytoplasmic BAG-1 had a significant survival advantage over patients whose tumor did not overexpress BAG-1, as illustrated in Figure 4*a*. Median survival could not be calculated for patients whose tumor overexpressed cytoplasmic BAG-1, since more than 50% of them were still alive at the time of analysis. The median survival of the patients whose tumors did not overexpress cytoplasmic BAG-1 was only 17.04 months ($p = 0.02$). Furthermore, patients whose tumors exhibited intense cytoplasmic staining for BAG-1 had a significant prolongation of median survival (46.02 months) compared with patients whose tumors exhibited negative or light staining for cytoplasmic BAG-1 (17.01 months), with a p value of 0.06 (Fig. 4*b*).

The influence of cytoplasmic BAG-1 expression on patient survival was also demonstrated by Cox regression analysis, which showed that intense cytoplasmic BAG-1 staining was correlated

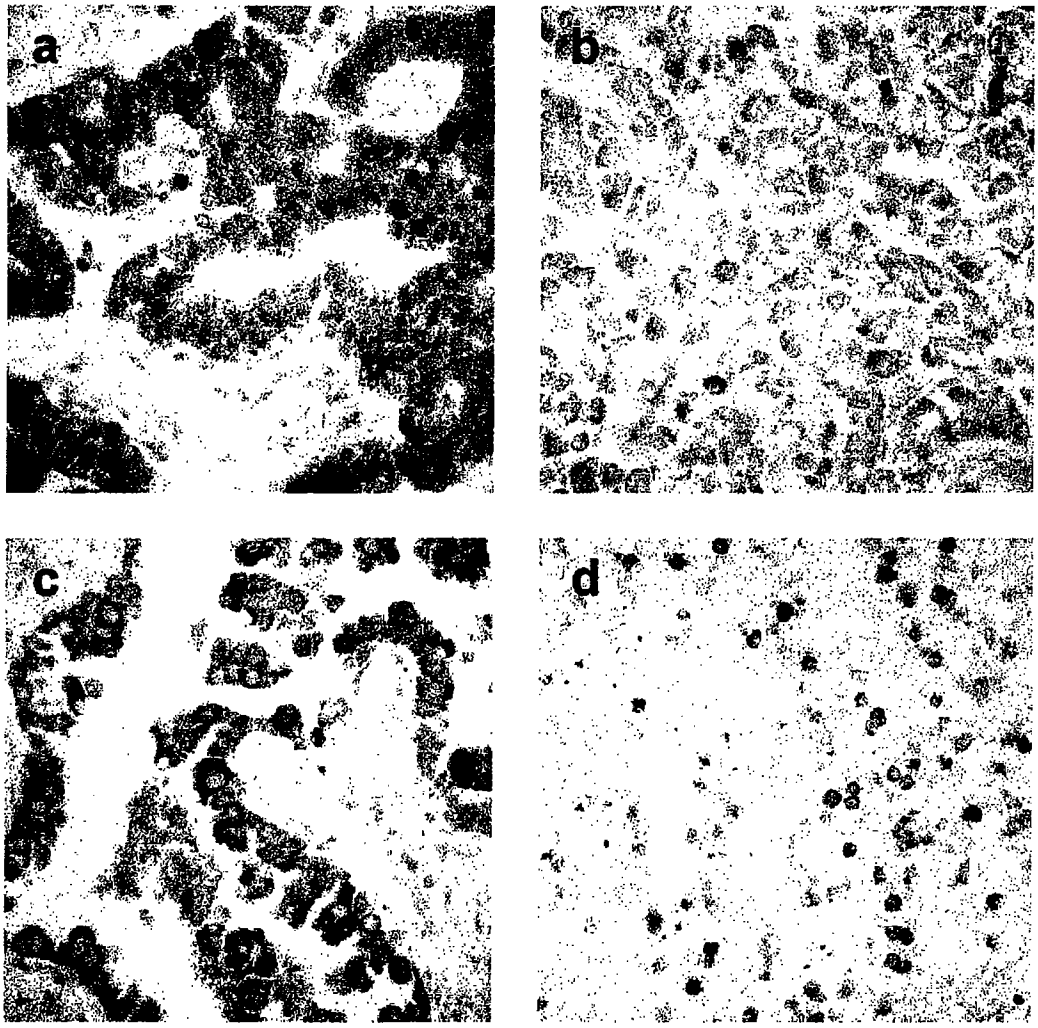

FIGURE 2 – BAG-1 immunohistochemical staining. (*a*) Adenocarcinoma, strong nuclear staining. (*b*) Squamous cell carcinoma, weak cytoplasmic staining. (*c*) Adenocarcinoma, strong cytoplasmic staining. (*d*) Squamous cell carcinoma, strong nuclear staining.

TABLE II – CORRELATION OF BAG-1 EXPRESSION WITH OTHER PROGNOSTIC FACTORS BY LOGISTIC REGRESSION

Factor	*p*-value
Age	0.71
Sex	0.84
Stage	0.35
Histology	0.43

TABLE III – BAG-1 EXPRESSION AND OVERALL SURVIVAL BY UNIVARIATE COX REGRESSION

Factor	Hazard ratio	95% CI	*p*-value
Age	1.04	1.0–1.07	0.02*
Sex	0.95	0.51–1.76	0.87
Histology	0.79	0.43–1.45	0.44
Stage			
I vs. IV	0.15	0.06–0.34	0.00*
II vs. IV	0.32	0.14–0.74	0.01*
III vs. IV	0.58	0.28–1.36	0.21
BAG-1+	0.53	0.30–0.95	0.03*

*$p < 0.05$.

TABLE IV – BAG-1 EXPRESSION AND OVERALL SURVIVAL BY MULTIVARIATE COX REGRESSION

Factor	Hazard ratio	95% CI	*p*-value
BAG-1+	0.58	0.31–1.04	0.07
Age	1.04	1.00–1.08	0.05*
Sex	1.84	0.89–3.80	0.10
Stage	1.84	1.39–2.43	0.00*
Histology	0.97	0.53–1.77	0.93

*$p < 0.05$.

with more reduction in the risk of death ($p = 0.05$; Table VI). When BAG-1 expression and overall survival were analyzed stage by stage, the correlation of BAG-1 overexpression and better survival seemed to be associated more with stage I disease ($p = 0.06$) than with stage II ($p = 0.75$), stage III ($p = 0.86$) and stage IV ($p = 0.34$) diseases (Table VII). We also analyzed the impact of nuclear BAG-1 expression on survival. Patients whose tumors overexpressed nuclear BAG-1 showed a trend toward longer survival compared witho patients whose tumors did not overexpress BAG-1, although the difference did not reach statistical significance ($p = 0.06$). Similarly, no difference in survival was detected in patients whose tumors overexpressed cytoplasmic BAG-1 compared with patients whose tumors overexpressed nuclear BAG-1 ($p = 0.06$; Fig. 5). Nine cases with both cytoplasmic and nuclear staining were excluded from the analysis. The median survival of patients whose tumors overexpressed cytoplasmic BAG-1 was not reached at the time of analysis, whereas the median survival of the patients whose tumors overexpressed nuclear BAG-1 was 30.1 months.

DISCUSSION

Recently, in a retrospective study on human breast cancer, we showed that increased BAG-1 expression correlated with poor survival in a multivariate analysis.[34] Furthermore, there was evidence that cellular localization of staining was important prognostically, as only patients with *nuclear* BAG-1 expression demonstrated the trend toward shortened disease-free survival and overall survival. We now report a survival *advantage* in NSCLC patients with BAG-1 expression. These findings may appear contradictory. However, the survival advantage attributed to BAG-1 expression in this report is specific for cytoplasmic staining. Our finding is consistent with a recent publication in which cytoplasmic BAG-1 expression correlated with better survival in patients with early stage breast cancer.[37] Therefore, the subcellular localization of BAG-1 expression appears to be of paramount importance when attempting prognostication using immunohistochemistry.

TABLE V – CYTOPLASMIC BAG-1 EXPRESSION AND OVERALL SURVIVAL BY MULTIVARIATE COX REGRESSION

Factor	Hazard ratio	95% CI	*p*-value
Age	1.04	1.00–1.08	0.03*
Sex	1.93	0.92–4.04	0.08
Stage	1.87	1.40–2.50	0.00*
Histology	0.90	0.50–1.64	0.74
BAG-1+	0.42	0.19–0.92	0.03*

*$p < 0.05$.

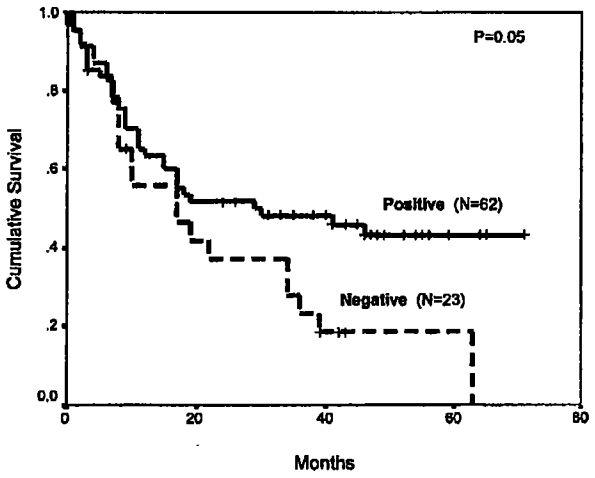

FIGURE 3 – BAG-1 expression and overall survival by Kaplan-Meier analysis.

The variable intracellular localization of BAG-1 expression has been well described previously. Extensive immunohistochemical analysis of BAG-1 expression in a number of normal human tissues has been completed.[36] Cytosolic, nuclear and mitochondrial expression were described. Within identical tissues, differential localization of BAG-1 appeared to be related to the degree of differentiation. For example, in epidermal tissue, BAG-1 appeared to redistribute from the nucleus to the cytosol during the differentiation of keratinocytes. Recently we also demonstrated the compartmentalization of BAG-1. BAG-1 was found to be generated as 4 isoforms by alternative translation initiation.[33] Our findings were subsequently confirmed by a separate study.[35] The p50 isoform is nuclear and the others are cytoplasmic. Alteration of subcellular localization of tumor suppressor gene has been suggested to play a role in the pathogenesis of sporadic endocrine pancreatic tumors[38] and follicular thyroid tumors.[39] Interestingly, the cytoplasmic BAG-1 p29 isoform was found to be completely absent from the cytoplasm of actively dividing human lung cancer cell lines. In the context of our current results, it is possible that increased p29 isoform expression in the cytoplasm of NSCLC results in slower tumor growth and may account for the favorable effect of intense cytoplasmic BAG-1 staining on survival. Finally, the significance of nuclear expression of BAG-1 in breast cancer (as opposed to NSCLC) may be related to BAG-1 effects on steroid hormone receptors that are present in the nuclei of breast cancer cells.[28]

Recent evidence supports Bcl-2 targeting of BAG-1 to intracellular membranes and organelles.[36] It is also known that Bcl-2 prolongs cell survival in concert with BAG-1.[25] It is possible that the expression of such closely related genes might have similar overall effects on prognosis. Reports of increased survival in NSCLC patients who exhibit increased Bcl-2 expression support this hypothesis.[14–17,21] However, inconsistencies in studies looking at the prognostic value of Bcl-2 in NSCLC exist,[18–20] and failure to consider intracellular localization of staining may be a contrib-

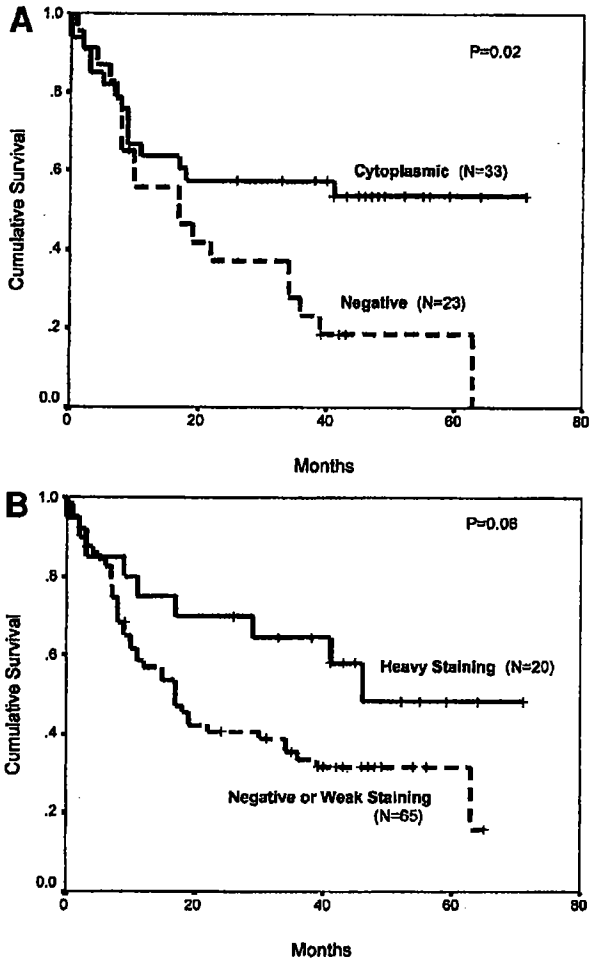

FIGURE 4 – (*a*) Cytoplasmic BAG-1 expression and overall survival by Kaplan-Meier analysis. (*b*) Intensity of cytoplasmic BAG-1 expression and overall survival by Kaplan-Meier analysis.

TABLE VI – INTENSITY OF BAG-1 STAINING AND SURVIVAL BY COX REGRESSION

	Hazard ratio	95% CI	*p*-value
Cytoplasmic BAG-1[1]			
1 or 2			
2 vs. 0	0.72	0.41–1.26	0.25
2 vs. 1	0.47	0.20–1.14	0.10
2 vs. 0	0.46	0.21–1.02	0.05*
Nuclear BAG-1			
1 or 2			
2 vs. 0	1.20	0.67–2.15	0.53
2 vs. 1	0.70	0.25–1.97	0.50
2 vs. 0	0.95	0.37–2.43	0.91

[1]Negative, weak or strong BAG-1 staining are designated as 0, 1 and 2, respectively.
*$p < 0.05$.

TABLE VII – BAG-1 EXPRESSION AND OVERALL SURVIVAL BY STAGE

Stage	Hazard ratio	95% CI	*p*-value
I	0.37	0.13–1.05	0.06
II	1.23	0.33–4.59	0.75
III	1.11	0.35–3.47	0.86
IV	0.48	0.11–2.16	0.34

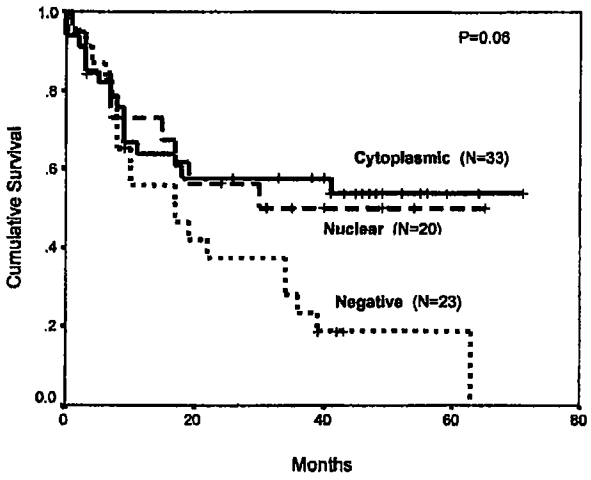

FIGURE 5 – Cytoplasmic and nuclear BAG-1 expression and overall survival by Kaplan-Meier analysis.

uting factor. In normal tissues, Bcl-2 has been found to reside primarily in the intracellular membrane.[36] Potentially favorable effects on host survival may occur when Bcl-2 is upregulated in the cytoplasm of tumor cells.

The prevention of apoptosis may lead to abnormal cell growth, tumorgenesis and tumor progression. How then might anti-apoptotic proteins such as BAG-1 and Bcl-2 provide a survival advantage to the patient? One possibility is that following establishment of a cancer cell line *in vivo*, the presence of anti-apoptotic activity may actually be advantageous to the host. At least 1 NSCLC study has addressed this question directly. In a retrospective study of 75 patients with a variety of NSCLCs, those tumors with enhanced apoptotic indices had a 1.9-fold risk of death ($p = 0.04$).[40] This seemingly paradoxic relationship between apoptotic activity and survival has been discussed previously with respect to anti-apoptotic Bcl-2 activity in follicular lymphoma (a notably indolent disease).[41] Plausible theories based on the balance between cell growth and cell proliferation are offered. Ultimately, however, it remains unclear how antiapoptotic activity in tumors may improve the survival of the host.

This is the first study demonstrating the prognostic significance of BAG-1 expression in NSCLC. Patients whose tumor overexpressed BAG-1 had a survival advantage, with a median survival of 30.10 months, compared with patients whose tumor did not overexpress BAG-1, with a median survival of 17.04 months ($p = 0.05$). In addition, intense cytoplasmic staining was correlated with a statistically significant increase in overall survival, independent of age, stage and histology. Furthermore, the magnitude of reduction in risk of death was impressive (hazard ratio = 0.42, $p = 0.04$). The findings were not limited to certain histologic or stage subgroups, and as such would be applicable in the clinical setting. Intracellular localization of gene expression may be an important consideration in other immunohistochemical applications. Our study strongly indicates that further large-scale retrospective and prospective studies are warranted to test the value of BAG-1 expression in guiding clinical management of nonsmall cell lung cancer.

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